Novel Therapeutics for Enteric Diseases

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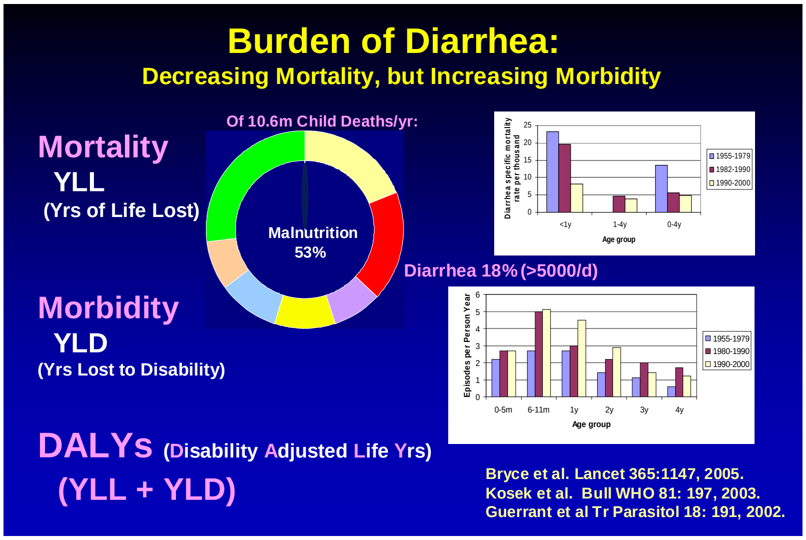
Measuring and Addressing the Burden of Disease
Richard Guerrant

Diarrheal disease remains a major infectious disease challenge that still accounts for an estimated 18% of the 10.6 million childhood deaths annually. Global statistics suggest that the direct mortality attributable to diarrhea has declined in the post-war years, however extensive epidemiological studies have shown that the impact of enteric disease extends far beyond direct mortality. Numerous enteric pathogens have been associated with diarrhea either alone, or in the form of co-infections. *Cryptosporidium parvum*, various pathogenic *Escherichia coli, Giardia lamblia,* Torovirus, Adenovirus, and HIV are all strongly represented, and many other diarrheagenic pathogens remain to be identified and characterized. Diarrheal disease causes structural damage to the intestine, malnourishment and adverse synergies with other diseases, for example by interfering with the absorbtion of anti-retroviral and anti-tuberculosis drugs. A recent study of HIV infected individuals in Gheskio, Haiti showed that diarrhea is the most common symptom among individuals with AIDS, and that diarrhea is predictive of mortality, highly active anti-retroviral therapy (HAART) malabsorbtion, and the presence of drug resistant HIV. In addition, severe, long-lasting deficits among Brazilian children have been associated with recurring diarrheal disease during childhood, including growth shortfalls, fitness impairment, cognitive impairment, and poorer school performance. Recent data, therefore, show that diarrheal diseases feed a cyclical poverty trap and are greatly underestimated as a factor in human health and welfare. The elderly who are especially susceptible to potentially problematic infection with *Clostridium difficile,* are also significantly impacted by diarrhea, which again leads to malabsorbtion of nutrients and medications.

Genetic evidence points to diarrheal disease applying evolutionary pressure in the selection of a protective balanced polymorphism. The ApoE4 allele, also known as an 'Alzheimer's disease gene' significantly protects children with a severe burden of diarrhea against the cognitive impairment seen among controls. Oral rehydration therapy can be supplemented with alanine, arginine, vitamin A, and zinc to improve the repair of intestinal epithelia in children with persistent diarrhea, individuals with HIV, and the elderly. Highly significant improvements have been seen in the proportion of HIV-positive patients with improved antiviral drug absorbtion.

- Key research needs
  - Broader epidemiological information on the worldwide cost, etiology, and incidence of diarrheal disease
  - Development of interventions such as vaccines, drugs, probiotics, or enhanced ORT
  - Further exploration into ORT supplementation to enhance recovery of intestinal mucosa
  - Further discovery on protective balanced polymorphisms to indicate potential research avenues for the development of therapeutics
  - Full assessment of the long-term impact of diarrhea on human health, development, and productivity
- Other needs
  - Establish diarrhea control efforts for individuals on HAART, anti-tuberculosis, and anti-cancer drugs

- Provide trans-national bodies with more accurate data showing the significance of diarrheal disease to support more appropriate allocation of research, epidemiology, and intervention efforts.



Enteric Diseases and Antimicrobial Resistance
Shaohua Zhao

Based on the FoodNet 1996-1997 study and other sources, the Centers for Disease Control and Prevention (CDC) estimated 211 million acute gastroenteritis, resulting in 900,000 hospitalizations, and over 6,000 deaths each year in the United States. Among the gastroenteritis, foodborne illness was a significant attribution. It is estimated that 76 million of foodborne illesses, 323,000 hospitalizations and 5,200 deaths each year (Mead et al. 1999). Over 90% of foodborne illnesses are caused by microorganisms. Among the bacterial agents, *Campylobacter* and *Salmonella* account for the greatest number of both estimated and reported causes of foodborne disease, followed by *Clostridium perfringens, Staphylococcus aureus*, various *Escherichia coli* pathotypes, *Shigella* spp., and *Listeria monocytogenes.* Studies have shown that antimicrobial-resistant non-typhoidal *Salmonella* causes treatment failures, complications, increased virulence, elimination of competing intestinal flora, pathogen overgrowth, increased likelihood of hospitalization, longer hospitalization, and an increased likelihood of death.

In 1996, the National Antimicrobial Resistance Monitoring System (NARMS) was launched as a collaborative effort among the Food and Drug Administration, CDC, and the U.S. Department of Agriculture, to document the emergence of antimicrobial resistant pathogens in the United States. A study by NARMS in 2002-3 showed that multi-drug resistance was widespread in *Salmonella* strains isolated from food animals on the farm, and raw retail meats. Without question, the transmission of antimicrobial-resistant pathogens from animal husbandry and the environment to humans represents a complex phenomenon, involving the management of human sewage, animal manure, clinical waste, and the disposal or recycling of other farm and slaughterhouse-derived material of animal origin. The role of therapeutic and sub-therapeutic use of antimicrobials as well as the use for growth promotion in the emergence and transmission of antimicrobial-resistant pathogens to humans has been described in detail as an unfolding public health concern. One example described the introduction and dissemination of streptothricin resistance in the former East Germany. When introduced on the farm to treat pigs in 1982, there was no streptothricin resistance noted in the animal or human microbial flora. By 1983 resistance was detected among *E. coli* strains isolated from pigs and in farm personnel. By 1984 resistance had spread to farm families, and in 1985 was found in the community in the form of streptothricin-resistant *E. coli* and as drug-resistant urinary tract infections. Lastly, in 1987 streptothricin resistance was found in the closely-related enterobacteriaceae *Shigella sonnei.*

Much evidence shows that removal of specific antimicrobials from the agricultural arena leads only to a slow diminution of resistance; that drug resistance is the rule, and not the exception; and that resistance increases from low levels to high levels, through an intermediate stage. This was seen with beta-lactam resistance in *Streptococcus pneumoniae*, and fluoroquinlone resistance in *E. coli*. In addition, the acquisition of resistance by one antimicrobial commonly expedites the acquisition of additional resistance genes.

Clearly, treatment of chronic enteric infection and re-infection with antibiotic-resistant organisms cannot represent a successful long-term strategy in the face of the rapid development of resistance, and the inevitable decrease in intestinal fortitude caused by unbalancing the normal protective intestinal flora. Furthermore, the timeline for antimicrobial drug development from discovery to product launch is approximately 9.5 years, which compares unfavorably to a few months or a year that it takes after launch for the first examples of resistance to be seen in human clinical isolates. In the likely absence of ‘prudent guidelines’ or more determined efforts, the problems of antimicrobial resistance will inevitably lead to further dissemination of incurable infections.

- RESEARCH NEEDS:
  - Improved surveillance of susceptibility and resistance is needed both nationally and internationally. Very little is known about the role of international policies and the global spread of resistance from countries with liberal animal husbandry or prescription policies, to countries with more restrictive policies.
  - Research into novel approaches to the development of antimicrobial compounds that elude resistance is urgently needed
  - Improvements in administration are needed, such as short-term, narrow spectrum, high dose therapy
  - Increased education on appropriate use is needed for physicians, veterinarians, clinical labs, agricultural producers, pharmaceutical companies, and policy makers worldwide.

Mechanisms of Bacterial Diarrheagenesis
Jim Kaper

Wide though it already is, the range of known bacterial agents of diarrhea is continually expanding with improved surveillance, epidemiology, and molecular diagnostics. A brief but by no means comprehensive list of powerful diarrheagenic enteric bacterial pathogens includes *Vibrio cholerae, V. parahaemolyticus, Shigella dysenteriae* Type 1, *S. sonnei, S. flexneri, S. boydii, Salmonella* Typhi, the non-typhoidal *Salmonella* serovars, *Campylobacter jejuni, C. coli, Clostridium difficile,* enterotoxigenic *Bacillus fragilis, Listeria monocytogenes, Aeromonas hydrophila, Yersinia enterocolytica, Y. pseudotuberculosis,* and a plethora of highly significant *Escherichia coli* pathotypes. These pathogens utilize an extensive array of virulence strategies and factors to generate the infected diarrheal discharge that mediates their transmission to the next host. Understanding the mechanistic basis for pathogenesis provides an excellent basis from which to develop therapeutic approaches that either mitigate the physiological damage to the host or inhibit the ability of the pathogen to express virulence.

The enterotoxins were the first characterized due to their dramatic specific activity. A mere 25ug of purified cholera toxin administered orally caused 20 liters of diarrhea in two volunteers. Other mechanistic categories include cell-destroying cytotoxins, the production of inflammatory mediators, effacement of the epithelium, increased intestinal permeability, epithelial cell invasion, and stimulation of the enteric nervous system. Cholera toxin (CT) is expressed with a neuraminidase enzyme that increases the density of the GM1 ganglioside toxin receptor. In addition, CT is co-regulated at the genetic level with the toxin co-regulated pilus (TCP) bacterial colonization factor. CT stimulates chloride secretion through the cystic fibrosis transmembrane regulator (CFTR) and inhibits sodium uptake by increasing cAMP production via the ADP-ribosylation of the Gs protein. In addition, CT causes high levels of prostaglandins in human jejunal fluid, a phenomenon associated with secretory diarrhea in animal models. The diarrheagenic capacity of *V. cholerae* is not fully explained by CT. Isogenic strains lacking the genes encoding CT remain capable of causing significant non-cholera diarrhea, mean volume 0.9 liters, in up to half of orally-infected volunteers, compared to a mean volume of 7.3 liters of diarrhea in volunteers fed with the fully virulent parent strain. Fecal lactoferrin levels in volunteers fed initial reactogenic *V. cholerae* vaccine strains indicate that a high degree of intestinal inflammation occurs in individuals fed strains lacking CT.

The remarkable range of *E. coli* pathotypes present a complex admixture of virulence factors in permutation and combination. The enterotoxigenic *E. coli* (ETEC) of travelers' and pediatric diarrhea are primarily characterized by the production of an enterotoxin analogous to the CT in *V. cholerae.* Additional ETEC toxins include the heat stable (ST) enterotoxin that activates guanylate cyclase. The heat stable A (STa) enterotoxin comprises only 18 amino acids, six of which are cysteine residues. STa acts at the cell surface by binding the membrane-spanning guanylate cyclase and activiating the conversion of GTP to cGMP, which results in chloride secretion, inhibition of Na absorption, and net fluid secretion. STa is not internalized, and is faster acting than CT. Homologues of STa have been found in *Yersinia* and *Vibrio.* In addition to toxins, there are over 20 adherence factors identified among various ETEC strains. The enteroaggregative *E. coli* that play such a significant role in persistent low-grade diarrhea in children living in developing countries express enterotoxins (Pet, EAST-1, and ShET-1) and inflammatory mediators (flagellin and the AafB pilus tip adhesin/invasion).

Enterotoxigenic *Bacillus fragilis* secrete a 20kDa zinc metalloprotease toxin called BFT that stimulates secretion in ligated intestinal loops of lambs, rats and rabbits. BTF also stimulates chloride secretion in polarized human intestinal cells, and IL-8 secretion via MAP kinases and a tyrosine kinase-regulated nuclear factor kB pathway. The protease activity of BFT appears to cleave the E-cadherin protein of epithelial zonula adherens.

Vibrio parahaemolyticus, the most common cause of seafood-associated bacterial gastroenteritis, expresses the thermostable direct hemolysin (TDH), an enterotoxin that induces calcium-dependent intestinal chloride secretion. *Campylobacter jejuni* pathogenesis is poorly understood, however infection causes an acute inflammatory enteritis. The cytolethal distending toxin (CDT) blocks cell division at the G2 stage, preventing mitosis, thereby possibly preventing epithelial cell replacement and persisting bacterial colonization of epithelia. Like ETEC, CDT also appears to induce IL-8 release from intestinal epithelia.

Clostridium difficile has recently extended from antibiotic-associated diarrhea and colitis to become a major source of health-care associated morbidity and mortality. *C. difficile* expresses two large toxins, A and B (respectively 308kDa and 270kD), which both appear to glucosylate and inactivate Rho GTPases to affect cytoskeletal structure and cell signaling. Disruption of actin may increase the permeability of tight junctions. In addition, the toxins A and B appear to trigger inflammatory responses involving a range of cell types in the lamina propria, including neurons, mast cells, and macrophages, which in turn stimulate neutrophil recruitment and the release of IL-8.

The potent Shiga toxins 1 and 2 (Stx1 and Stx2) of the enterohemorrhagic *E. coli* (EHEC) bind the Gb3 cellular receptor, depurinate rRNA, and thereby inhibit protein synthesis. Stx also directly damages glomerular endothelia, causes apoptosis, and induces cytokine production, including TNF-alpha, IL-1Beta, and IL-6. Stx kills the absorptive villus cells while preserving secretory crypt cells, thereby changing the absorptive balance.

An additional mechanism of diarrheagenesis involves up-regulation of the galanin-1 receptor, which is widely distributed in enteric nerve terminals. Activation of galanin-1 receptor causes colonic chloride secretion, and is a mechanism reported in EHEC, enteropathogenic *E. coli* (EPEC), *Salmonella,* and *Shigella,* but not normal flora *E. coli.*

The type three secretion system (TTSS) is a needle-like apparatus possessed by *Salmonella, Shigella, Yersinia, V. parahaemolyticus, Aeromonas, Pseudomonas,* various *E. coli* pathotypes, *Chlamydia,* and plant pathogens. The TTSS functions to inject proteins into the cytosol of host cells; these proteins mediate epithelial invasion, systemic survival, enterotoxic activity, and other functions. The SopB and SopD proteins secreted through the TTSS of *Salmonella* induce basolateral secretion of IL-8. SipA induces apical production of pathogen-elicited epithelial chemoattractant (PEEC) that directs polymorphonuclear leukocytes across epithelia. In addition, SopB is an inositol polyphosphate phosphatase that causes a transient increase in IP4, production of prostaglandins, translocation of PMN, which secrete 5'AMP, resulting in activation of adenosine receptor.

The four species of *Shigella* share several pathogenic mechanisms, although the *S. dysenteriae* Type1 produces Shiga toxin, is the most virulent, and is capable of causing epidemic dysentery with severe mortality. The ShET1 and ShET2 enterotoxins may account for early (18-24 hour) watery diarrhea, which is followed by severe bloody diarrhea caused by the Shiga toxin (days 2-7). Shigellae pathogenesis is mediated by TTSS effectors (IpaA, B, C, D, and IpgD) that allow invasion of M cells. Shigellae invades epithelia through the basolateral route, then spreads from cell to cell via actin polymerization mediated by IcsA. Apoptosis of macrophages and other cells is mediated by caspase-1 activation, which releases inflammatory mediators. Epithelial cells secrete IL-8, attracting PMNs. In addition, the Nod1/Nod2 intracellular sensors recognize bacterial peptidoglycan, activating NF-kB.

Many of the pathogenic factors involved in epithelial colonization, cell invasion and toxicity are present on coordinated, coregulated, or physically linked genetic elements. Significant cell-to-cell transmission of mobile genetic elements has resulted in pathogens possessing a mosaic of elements derived from external sources. The pathogenicity islands, well illustrated by the locus of enterocyte effacement (LEE) in EPEC and EHEC, represent a family of linked pathogenicity factors. Intriguingly, the LEE (and many other aspects of bacterial pathogenesis) is regulated by quorum sensing. It is possible that pathogenic *E. coli* such as EHEC use the quorum sensing system of normal enteric *E. coli* to recognize the appropriate niche in which to activate LEE and activate TTSS, adhesion, and invasion processes.

The broad scope of enteric bacterial pathogens and the variety of mechanisms that they utilize to infect, proliferate, and transmit clearly represents only the surface of the potential virulence strategies available to enteric pathogens. Increasing evidence shows that pathogens will utilize a combination of approaches and often possess a redundancy of mechanisms which allow them to persist and re-infect, even after the individual has recovered. There is little doubt that the generation of novel pathogenic mechanisms represents a formidable challenge to therapy, however the following understudied areas of research clearly are of importance in mitigating the severest manifestations of disease.

RESEARCH NEEDS:

- It is necessary to relate tissue culture and animal models to human diarrhea
- More information is needed to relate intestinal TLR and other components of the innate immune system to how they interact with enteric infections
- More information is needed on intestinal inflammation, in response to bacteria, and the role of bacteria in inducing chronic intestinal inflammation
- Further information on small molecule inhibitors of quorum sensing and TTSS may provide keys to novel therapeutics that will provide a low pressure on the development of resistance
- Improved diagnosis of enteric disease is needed so appropriate therapy may be initiated at the earliest moment

Mechanisms of Parasitic Pathogenesis
William Petri

The enteric parasites represent a major class of diarrheagenic pathogens for which few effective therapeutic options are available. The World Health Organization estimates that approximately 50 million people worldwide suffer from invasive amebiasis each year, resulting in 40 to 100 thousand deaths. Serologic studies in Mexico demonstrated antibody to *E. histolytica* in 8.4 % of the population. The annual incidence of amebic liver abscess is 21 cases/100,000 inhabitants in Hue City, Vietnam. A prospective study of preschool children in a slum of Dhaka Bangladesh has shown a 9% annual incidence of *E. histolytica* - associated diarrhea or dysentery. In the United States amebiasis is especially a problem in travelers to and in recent immigrants from the developing world, as well as in men who have sex with men. *Entamoeba histolytica* binds intestinal epithelia through a Gal/GalNAc lectin encoded by the *hgl* and *igl* genes. GalNAc-containing neoglycans are potent inhibitors of amebic adherence which may play a role in preventing or reversing colonization at an early stage in the infective cycle. Host cell death soon follow Gal/GalNAc lectin-induced adherence through apoptosis via activation of the host caspase 3 system. Current therapies for asymptomatic infection include iodoquinol, paromomycin, or diloxanide furoate. Invasive amebiasis is presently treated with metronidazole or tinidazole. The degree to which drug resistance in amebiasis has developed is not known at present. Recent advances, including the sequencing of the genome and understanding of molecular pathogenesis, have identified novel drug targets including a family of receptor kinases, cysteine proteinases, alcohol dehydrogenase and Fe-S complex proteins. It would be prudent to invest in the development of new therapies since there is only one class of agents that is currently known to be effective for invasive amebiasis.

Giardia lamblia is the most common parasite identified in stool samples of individuals in the USA, present in ,4% of stool specimens submitted to clinical laboratories. The disease is quite common in developing countries as well, especially in urban slums where a substantial number of children are infected. Water- and food-borne transmission are the most frequent mechanisms of spread, with person to person spread important in day care settings and among sexually active homosexual males. *Giardia lamblia* pathogenesis is similarly poorly defined. Trophozoites of *G. lamblia* can be seen adherent to the intestinal epithelium on small bowel biopsies, and the organism is known to undergo antigenic variation of a family of surface proteins called VSPs. Therapy utilizes metronidazole, tinidazole, or nitazoxanide, with second line agents such as paromomycin, furazolidone or quinacrine sometimes required due to failure of first line therapy.

The spore-forming protozoa (cryptosporidia, cyclospora, isospora, and the microsporidia which are now classified as fungi) are named according to the infectious spore form of the parasite which is spread in a fecal–oral manner. After ingestion of spores from contaminated food or water, sporozoites are released which invade into the intestinal epithelium where they replicate intracellularly. In humans with a normal immune system, infection with an intestinal spore-forming parasite leads to a self-limited diarrhoea. Treatment is usually not required. CD4 counts at >200 mmm3 are associated with persistent diarrheal infection with cryptosporidia, isospora, cyclospora and microsporidia. In the USA, C. parvum is the most frequently identified spore-forming parasite in AIDS patients with chronic diarrhoea, whereas in developing countries

I. belli and Cyclospora cayetanensis are also frequently identified. *Cryptosporidium parvum* and *C. hominis* can be treated in non-HIV infected with nitazoxanide, but there is no treatment for cryptosporidiosis in the HIV infected. The recent sequencing of the cryptosporidial genome has demonstrated the absence of many metabolic pathways, explaining in part the prior difficulty in developing therapeutics, and at the same time identifying promising therapeutic targets.

The microsporidia include twelve species known to infect humans, the most significant of which are *Enterocytozoon bieneusi* and *E. intestinalis* which can infect through inhalation or oral ingestion of spores. Sporozoites infect epithelial cells of the intestine or respiratory tract. Intestinal *E. bieneusi* is usually treated with fumagillin (not available in the U.S.), and intestinal *Encephalitozoon intestinalis* with albendazole. Symptoms can be ameliorated with octreotide. Disease appears to be confined to the immunosuppressed, although additional studies are needed to delineate its contribution to diarrheal disease in non-immunocompromised individuals. Cyclospora represents a new pathogen in North America. Over 1000 cases have been diagnosed in the US since the summer of 1996. Cyclospora can cause several weeks of diarrhea in immunocompetent patients. In a study among Haitian AIDS patients, 10% of those with diarrhea were chronically infected with Cyclospora. Cyclospora is treated with trimethoprim-sulfamethoxazole or ciprofloxacin. The life-threatening consequences of diarrhea in HIV/AIDS patients necessitate long-term suppression of parasites.

- Research Needs:
  - A better understanding is needed of the metabolic processes and pathways available to parasites
  - The interaction between parasites and host cells (microbial adherence, invasion, and host cell killing) needs to be better characterized
  - Further understanding of diarrheagenic processes are needed
  - Epidemiologic studies to estimate the contributions of the enteric parasites to disease are needed.

Molecular Basis of Cholera Toxin Action in Mammalian Cells: Modulation of Toxin ADP-ribosyltransferase Activity by Endogenous ADP-ribosylation Cycles

Joel Moss, Jianfeng Zhu, Shunya Oka, and Jiro Kato

Cholera toxin, produced by Vibrio cholerae, is responsible, in large part, for the devastating diarrhea characteristic of cholera. The toxin exerts its effects on cells through the ADP-ribosylation of Gαs, the stimulatory guanine nucleotide-binding protein that activates adenylyl cyclase, resulting in increased intracellular cyclic AMP content, and the often life-threatening derangements in fluid and electrolyte fluxes that are characteristic of the disease (1). Mammalian cells contain NAD:arginine ADP-ribosyltransferases, which, similar to cholera toxin, catalyze the modification of arginine residues in proteins; the ADP-ribose-arginine bonds are cleaved by ADP-ribose-(arginine)protein hydrolases (ARH), which regenerate the free (arginine)protein, thus completing an ADP-ribosylation cycle (2). We identified three genes that encode proteins with ARH-like sequences (3). Only one of the protein products of these genes, ARH1, was capable of cleaving ADP-ribose-(arginine)protein (3). We hypothesized that ARH1, if capable of cleaving ADP-ribose(arginine)Gαs, synthesized by cholera toxin, might reverse or limit the effects of cholera toxin on intestinal cells. In ARH1, aspartate residues 60 and 61 are critical for activity; their replacement with alanine results in an ARH1 protein with <0.1% of wild-type activity (4). ARH1 knockout mice were generated by replacing the exon containing the critical aspartate residues with a neomycin cassette. The ARH1 knockout mice are viable. Effects of cholera toxin on fluid accumulation in intestinal loops were significantly greater in the knockout than in the wild-type animals. Effects of cyclic AMP, the downstream signaling molecule generated by cholera toxin-catalyzed ADP-ribosylation were not, however, different in intestinal loops of the wild-type and knockout animals. Cholera toxin-generated ADP-ribosylarginine content and Gαs modification were significantly higher in intestinal epithelial cells from the knockout than from the wild-type mice. Similar findings were obtained with knockout and wild-type cells in culture. Thus, the effects of cholera toxin were significantly influenced by cellular ARH1 activity, which could function as a modifier gene in disease and serve as a potential therapeutic target. Presumably, in patients with cholera, toxin activity overwhelms this novel host defense, leading to persistent activation of adenylyl cyclase and the diarrheal syndrome characteristic of the disease.

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Therapy: Current Status of Oral Rehydration

William B. Greenough, III

Therapy for enteric infections has been anchored on the success of oral rehydration. Although the global use of ORT is limited to approximately 50% of the need, three million lives are saved annually. In addition to the invaluable role for ORT in replacing fluid loss during childhood diarrhea, ORT is finding an increasingly important application in geriatric care.

The origin of ORT lay in balance studies, the understanding of solute-mediated transport, and the use of digestible polymers that increased co-transport without incurring an osmotic cost. An essential element in the development of ORT were studies on cholera patients undertaken at the Cholera Research Laboratory now the International Centre for Diarrhoel Diseases Research, Bangladesh (ICDDR,B) in Dhaka, and the Johns Hopkins ICMRT, Calcutta, India. The basis of ORT was the observation that providing glucose with water and salts greatly enhanced the uptake of water and solutes, and even though diarrhea continued unabated, the net result was a positive fluid balance. Standard glucose-based ORT replaces lost volume, but does not reduce diarrhea. The cotransport of sodium and glucose provides the means to replace fluid losses during intestinal inflammation or intoxication. The use of rice-based ORT increases efficiency of fluid absorption because starch, when digested, provides more glucose to intestinal cotransport sites at low osmotic pressure in the lumen.

There remain opportunities to improve ORT by adding proteins or peptides that could hasten recovery of intestinal epithelium while providing added cotransporting channels. In addition, additives could be included to reduce the quantity of diarrhea, decrease inflammation, or inhibit microbial pathogenesis. In addition, improving the taste of ORT would encourage more widespread adoption. Criteria for additives to ORT must include non-toxicity in high doses, low price, and broad accessibility. In addition, the taste should not detract from the acceptability of ORT, or work against the long-term efforts at advocacy for this remarkable therapy. Potential additives include zinc, histidine or glutamine containing proteins or peptides, lactoferrin, lysozyme (breast milk proteins), and gut flora restoratives.

ORT will play an increasing role in geriatric medicine. The case fatality rate from gastroenteritis increases steadily with age, reaching over 100 deaths per 10,000 hospitalizations. The elderly have a high risk from the complications of hypovolemia, due to a diminished circulatory reserve, blockage of arteries to vital organs, lowered thirst drive, reduced mobility and balance, diuretics and low salt diets, and the effects of drugs used to lower blood pressure. ORT remains a potent accessible and low-cost treatment that satisfies the essential replacement concept: to put back what is lost in quantity and composition. Nonetheless, the following research needs remain outstanding:

- Identify the maximum co-transporting substrates with the lowest osmolarity
- Identify the most effective additives
- Improve the taste of ORT
- Explore adjuvant effects that may improve response to oral vaccine or natural infection
- Development of K+ free ORT to minimize the risk of hyperkalemia in renal failure

- Maintain low cost and continue to advocate broader usage to reach the 50% who presently do not benefit from this remarkable treatment option.

Crofelemer in the Treatment of Secretory Diarrhea

Barry Quart, Pharm. D. & Steven King Ph.D.
Napo Pharmaceuticals

Crofelemer, previously known as SP-303, is a novel proanthocyanidin purified from the bark latex of the Amazonian Croton tree *Croton lechleri.* Worldwide patent applications for enteric coated capsules have been filed for its use as an inhibitor of secretory diarrhea. Clinical studies (conducted under ICH GCP) involving 1,400 patients in double-blinded, placebo-controlled studies have establish that the drug is well tolerated and effective in mitigating travellers' diarrhea, and the chronic diarrhea of HIV/AIDS.

Crofelemer is an inhibitor of the cystic fibrosis transmembrane regulator chloride channel, as evidenced by its activity on cell cultures, single cell patch clamps, single CFTR channels, and elaboration of mouse intestinal fluid secretion. No effect was found on adenylate cyclase activity, or cAMP, indicating that crofelemer acts directly on the known mechanism of cholera and enterotoxigenic *Escherichia coli* fluid secretion in humans, and presumably other agents of secretory diarrhea that use the CFTR mechanism of diarrheagenesis. Extensive preclinical toxicology studies have been undertaken, including 32-day rat studies, 30-day dog, 9 month dog, Ames test, rat micronucleus, chromosomal aberration, rat fertility, embryo/fetal studies in rats and rabbits, and both pre- and postnatal development in rats.

A Phase II double-blind, randomized, placebo-controlled trial conducted at Jamaica (122 patients), Mexico (49), and US Border Towns (13) evaluated placebo, 125 mg, 250 mg, and 500 mg of crofelemer in a four-armed study over 2 days of treatment and one day of observation. The efficacy endpoints used were TLUS72 (time after last unformed stool), proportion of patients who were no better or worse after 24 hours, and symptomatic improvement, including changes in urgency and abdominal pain. Significant improvements were seen with all doses compared to placebo control in TLUS72, and significant reduction in treatment failures were seen over placebo controls. Moderate/severe abdominal pain and urgency improvement was also seen in the 250 mg group. After the first 24 hours of treatment, 91.3% of patients receiving 250 mg vs 65.9% patients receiving placebo were partial or complete responders (p=0.003).

A Phase II double-blind, randomized, placebo-controlled trial in HIV-associated diarrhea administered 500 mg crofelemer or placebo to 51 patients with HIV/AIDS (25 placebo, 26 crofelemer). Significant reductions were seen in stool weight (p=0.008), stool frequency (p=0.04), and stool chloride concentration (p=0.04). In a Phase III study of crofelemer in HIV-associated diarrhea in 400 patients with HIV/AIDS, four arms comprising placebo, 250 mg tablets, 500 mg tablets, and 500 mg beads were compared. Initial analysis showed borderline significance (p=0.03 vs. required of p=0.015), however reanalysis of patients with significant diarrhea at baseline (defined as watery stool and urgency at baseline) showed significant differences for change in stool weight (p=0.01), stool frequency (p=0.02), and frequency of loose/watery stools (p=0.003). The supply of raw material for crofelemer production has been assured through a huge natural stock of croton, sustainable harvesting research and the addition of 300,000 of these fast-growing trees through reforestation programs. Over $1M has been invested in long-term supply efforts.

Present plans include commercial manufacturing, and a Phase II proof-of-concept trial for effectiveness against cholera, and a confirmatory Phase III trial in HIV-associated diarrhea in 2006-2007.

In conclusion, crofelemer offers a natural product inhibitor of secretory diarrhea through inhibition of the CFTR chloride transporter. Crofelemer is not an antimicrobial, and therefore does not drive the emergence of resistance, it does not inhibit motility, and therefore does not cause constipation or rebound diarrhea, and it is not systemically absorbed, reducing the potential for adverse drug interactions and toxicity. Future research should address:

- Demonstration of efficacy against cholera
- Evaluation of safety and effectiveness in children and infants
- Impact on normal intestinal flora
- Comparison between patients and controls to evaluation of seroconversion of individuals convalescent from travelers' diarrhea, so determine if crofelemer inhibits colonization, or if colonization proceeds and provides convalescent immunity.

CFTR INHIBITORS AS POTENTIAL THERAPY TO REDUCE INTESTINAL FLUID LOSS IN CHOLERA AND OTHER SECRETORY DIARRHEAS

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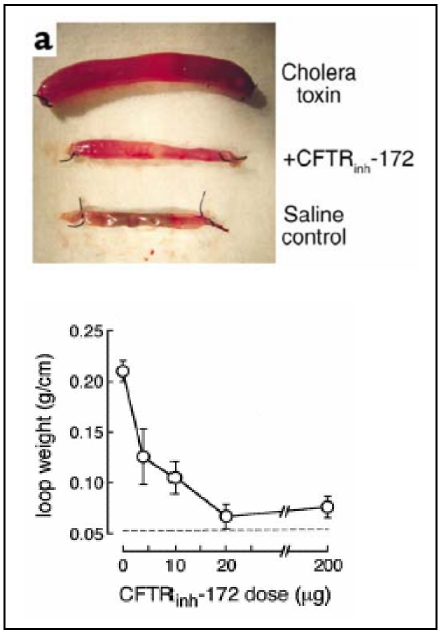
There is a need to develop a safe and effective treatment for secretory diarrhea induced by *Vibrio cholerae* and enterotoxigenic *E. coli* (ETEC). The molecular mechanism leading to diarrheas caused by these organisms is well understood. Both cholera and ETEC disease is typified by enterotoxin production which results in cAMP production. In addition, ETEC infection frequently results in liberation of stable toxin (ST) that activates production of cGMP. A cascade of events ensues, ultimately causing phosphorylation of the ABC transporter CFTR. Because this transporter is an integral chloride channel, phosphorylation increases chloride efflux that, in turn, actively drives sodium and water secretion into the intestinal lumen resulting in a form of diarrhea known as secretory diarrhea[1]. An analogous situation holds in cholera, where the toxin irreversibly activates the cAMP signaling cascade with attendant phosphorylation of CFTR. Activation of CFTR by ETEC and cholera toxin is directly related to the severity of the diarrhea seen in these two disease states. Accordingly, pharmacological inhibition of CFTR has been shown to be a strategy for the attenuation of secretory diarrheas in animal models of cholera and ETEC infection[2-4] and is therefore a strategy for the treatment of travelers diarrhea[5] (TD); a transient secretory diarrhea most commonly caused by infection with ETEC[6, 7]. CFTR is expressed on the apical surface of epithelial tissues including bronchial passages, pancreatic ducts, sweat ducts and the gastrointestinal tract[8]. Cystic fibrosis patients and animal models have decreased gastrointestinal secretory activity[9] and both CF patients and CFTR knockout mice do not develop diarrhea in response to heat stable enterotoxins[10].

Using high-throughput screening (HTS), Alan Verkman (University of California, San Francisco) has identified two classes of potent CFTR inhibitors with potential as anti-diarrheal therapy. The first class discovered was thiazolidinones and one of these is the small-molecule CFTR inhibitor, 3-[(3-trifluoromethyl)phenyl]-5-[(3-carboxyphenyl) methylene]-2-thioxo-4-thiazolidinone ($CFTR_{inh}$-172)[4]. The later compound class discovered was the glycine hydrazide class which, by electrophysiology studies, appears to block the CFTR ion channel on the lumen-facing surface[11]. The most potent compound from both these classes has potency in the low micromolar IC50 range with the value varying somewhat depending on the type of assay and manner in which it is executed.

Both of these compounds were originally discovered through the use of a cell-based FRT assay in which CFTR activity is pre-stimulated by addition of forskolin (cAMP agonist), IBMX (phosphodiesterase inhibitor and direct activator) and apigenin (flavone-type direct activator). The FRT cells co-expressed the <u>Y</u>ellow <u>F</u>luorescent <u>P</u>rotein (YFP) -based Cl^- / I^- sensor YFP-H148Q that provided a quantitative fluorescence read-out of inhibition potency. After CFTR pre-stimulation and compound addition, cells were subjected to an inwardly-directed I^- gradient to drive I^- influx and produce decreasing fluorescence. These molecules were also assayed using the techniques of short circuit current measurements in Ussing chambers and whole cell patch clamp electrophysiology with various membrane and cell types. This provides another route to measure inhibitory potency of these compounds.

An *in vivo* model was used to test the compound's antidiarrheal efficacy in cholera toxin-induced diarrhea. Both intestinal fluid absorption and secretion were tested in a closed intestinal loop mouse model[12]. Intraperitoneal administration of $CFTR_{inh}$-172 at a dose that strongly inhibited cholera toxin-induced intestinal fluid secretion (20 µg) did not alter the rate of fluid absorption (measured at 30 min) compared to controls.

Both the thiazolidinone and glycine hydrazide CFTR inhibitor block cholera toxin-induced fluid secretion in closed intestinal loops. The figure below summarizes a $CFTR_{inh}$-172 dose-response study in mice in which a single dose of inhibitor was administered by intraperitoneal injection just after infusion of cholera toxin into closed intestinal loops. Similar results were seen for the glycine hydrazide compound.



Basal intestinal fluid content was near zero as measured in non-cholera toxin injected loops. $CFTR_{inh}$-172 inhibited fluid accumulation in cholera toxin-injected intestinal loops by ~90%, with 50% inhibition at ~5 µg $CFTR_{inh}$-172. The duration of inhibition was measured as in the dose-response study, except that a single 20 µg dose of $CFTR_{inh}$-172 was administered at different times before or after cholera toxin. Good efficacy was seen for time points up to 3 hours before and after cholera toxin challenge. These experiments were carried out with both mice and rats and fluid accumulation was stimulated with both cholera toxin and heat stable ETEC toxin. In all cases the CFTR inhibitors tested reduced fluid accumulation.

The CFTR inhibitor $CFTR_{inh}$-172 is under development as a candidate antidiarrheal drug treatment. Toward that end, a range of development activities have been conducted including continued efficacy studies, single and multi-dose toxicology experiments, pharmacokinetic evaluation, distribution studies, formulation research, specificity evaluation, metabolism, bioanalytical and analytical method development, stability, manufacturing development, and solubility and permeability evaluation

Significant work remains to be done in the evaluation and development of CFTR inhibitors for the treatment of secretory diarrhea. Our work to date has been very encouraging and Active Pass continues to develop these molecules toward clinical trials, targeting the first clinical evaluation in 2006.

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Inflammation
Malgorzata Simm

Inflammation is a cellular response to an injury or abnormal stimulus caused by a physical chemical or biological substance and represents a major component of the human innate response to microbial pathogens.

Pathogen invasion in the gut elicits expression of a range of inflammatory mediators including MCP-1, IL-8, Gro, histamine, IL-1 β. This induction of expression of proinflammatory molecules is mediated through bacterial lipopolysaccharide or its N'terminal-fMet-Leu-Leu-peptides and leads to activation of NF-κB which in turn induces the release of TNF-□ and evokes numerous responses from immune cells and surrounding tissues. Although in general the secretion of TNF-□ is beneficial for rapid activation of immune responses, the prolonged activation of this cytokine favors pathological effects leading to destruction of intestinal epithelium, fluid loss, increased diarrhea and degradation of the structural defenses of the gut.

Different components of bacterial antigens induce inappropriate activation of NF-κB in infected tissues. For example LPS plays a major role during infection by *Shigella flexneri*, *Yersinia enterocolytica Salmonella enterica* and *Shigella dysenteriae*. However, additionally to LPS mediated activation, some strains employ also their toxins to induce NF-□B/DNA binding, as was found for invasin in *Yersinia enterocolytica* or factors of the Type III Secretion System and SipB in *Salmonella enterica* Typhimurium infections. Also the Shiga toxin of *Shigella dysenteriae* Type 1 mediates the expression of TNF-α through inappropriate NF-□B activation.

Currently available therapies are directed to eradication of pathogens, resulting in cessation of virulence factor expression, and concomitant decline in the expression of inflammation factors. However new approaches are needed to target pathogens through improving the host cellular responses to the invasion and making the target cells unavailable for infection. The HIV-1 Resistance Factor (HRF) is a protein expressed by CD4+ T cells that have been induced to resist viral infection. However, we found that exposure to soluble products of HRF-producing cell line impeded NF-□B/DNA binding in human macrophages induced by LPS from several species of bacteria including *Vibrio cholerae, Salmonella minnesota*, and *E. coli* and resulted in impaired TNF-□ responses to these organisms suggesting that HRF might have a broad activity against organisms whose pathogenesis is linked to NF-□B activation.

Subsequent studies on the mechanism of HRF-mediated inhibition of NF-□B/DNA binding showed that HRF interacts with p50 component of NF-□B dimer after it enters to the nucleus, but before its binding to cognate DNA motif and that this interaction impedes the formation of an NF-□B-DNA complex required for the promotion of transcription.

Taking altogether HRF is a novel NF-κB antagonist, a human protein secreted by CD4+ T-cells and offers some promise as a broad spectrum immunotherapeutic to interrupt the inflammatory pathway associated with infection by several different pathogens such as *Salmonella, Shigella, Campylobacter,* and HIV-1.

Gene Polymorphisms that Predispose to Infectious Diarrhea
Pablo Okhuysen

The triangle of pathogen, host, and the environment encompasses age, immunity, race/ethic factors in the host; travel, migration, ecological niches, and industrialization in the environment; and mutations, acquisitions, and losses of genetic elements in the pathogen. Human genetics encompasses functional variation in the human genome, which has evolved to facilitate defense against infectious pathogens. In most cases, the susceptibility to infection is polygenic, rather than being limited to just one or a few genes. It appears that genes expressed in response to infection evolve at a higher rate, and that the genomes of both pathogen and host evolve in response to a changing environment. The magnitude of the effect is illustrated by familial studies where close physical proximity in a common environment, for example in sibling studies, has shown that the genetic contribution increases the risk by 1.5 to 5 times. There are also inter-racial and inter-population differences. Susceptibility to infection is inherited, and the early death of a parent from infectious disease carries a six-fold risk of a similar fate for the offspring. The phenomenon is more clearly demonstrated in chronic parasitic, fungal, and mycobacterial infections. Furthermore, genetics determines the severity of infection, in addition to susceptibility. The reasons for studying host genetics include understanding pathogenesis, resistance mechanisms, risk prediction and behavior modification, risk assessment, improved use of vaccines and therapeutics, and the use of 'genetic profiling' to inform individualized prevention and treatment of diseases, rather than identification of new therapeutic targets and a deeper understanding of pharmogenomics.

Approaches to studying human host genetics and disease susceptibility include association studies in case control studies and mapping genes in susceptible mice, combined with a search for homologs in humans and genetic linkage studies in families, where genes can be identified without knowing their function a priori.

A useful model to study the contribution of genetics to susceptibility to gastrointestinal infection is Travelers' diarrhea which affects 40% -60% of individuals at risk, including considerable morbidity among troops and tourists both in acute disease and in terms of chronic complications, such as post-infectious irritable bowel syndrome, reactive arthritis and others.

Bacterial pathogens account for 85% of travelers' diarrhea cases, of which the etiological agents are most commonly enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), *Shigella, Campylobacter jejuni, Salmonella, Aeromonas, Plesiomonas,* and *Vibrio cholerae*. *Cryptosporidium* and *Giardia* are the most common parasites.

ETEC is a non-invasive cause of secretory diarrhea ETEC elaborates two major toxins, namely the labile toxin (LT, a homolog of CT, expressed by cholera toxin), which bind gangliosides GM1 and GD1b, and the heat stable toxins (ST), which bind and activate guanylate cyclase.

EAEC causes secretory and inflammatory diarrhea primarily in children, patients with AIDS, travelers, and ii associated with foodborne outbreaks of disease. When incubated with cultured human intestinal epithelial cells of the HEp-2 line, there is a characteristic 'stacked brick' pattern that is associated with the presence of a 60 MD plasmid. EAEC possesses the several putative virulence factors, namely the EAEC enterotoxins, which are distinct from LT or CT and include the plasmid-encoded toxin (Pet), heat stable enterotoxin (EAST-1) as well as other virulence factors such as, *Shet-1, Shet-2;* dispersin (*asp2)*, flagellin (*fliC*), mucus biofilm, and the adherence fimbriae AAF/I and AAF/II. The receptor for a subset of EAEC adherence is Decay Accelerating Factor (DAF, CD55), which is the receptor for Dr adhesins such as AAF/II. CD55 is known to down-regulate complement activity. In addition, CD55 contains 10 epitopes of the Cromer Blood groups which possess 40 single nucleotide polymorphisms. Furhtermore, Dr+ organisms bind to CD55 at SCR 2,3,4. The infection of the intestinal epithelia by EAEC results in increased levels of IL-8 via NF-κβ activation, which results from elaborateinteractions between flagellin and TLR5.

Gastrointestinal infection can be conceptualized into three stages, first during the earliest stage of susceptibility, pili, adhesins, and colonization factors interact with host factors such as receptors, innate immunity components, opsonins, defensins, collectins, TLRs, and organisms are subject to pre-existing immunity. The injury phase is characterized by expression of virulence factors, and host responses encompassing inflammatory cytokines, chemokines, prostaglandins, kinins, and functional activation of targets. The recovery/healing phase is characterized by anti-inflammatory cytokines, growth factors, and specific humoral, cellular, and secretory immunity. At each level the response can be impacted by host genetic variation.

The study objectives were to conduct a SNP candidate association study to determine the susceptibility and severity of travelers' diarrhea, the fecal cytokine response to infection, the relative contribution of host pathogen factors have on disease, and evaluate post-travel chronic diarrhea and IBS-like symptoms. The study involved US travelers to Guadalajara, Jalisco and Cuernavaca, Morelos, Mexico in 2002-05. Volunteers were enrolled within 72 hrs. of arrival, and blood was collected both on arrival and departure for serology and DNA studies. During the stay, subjects recorded their symptoms in a diary and they were instructed to contact the clinic at any time of day or night in case of diarrhea. The laboratory studies included evaluation of diarrheal stools for mucus, fecal leukocytes, and occult blood, and the presence of *Shigella, Salmonella, Campylobacter,* ETEC (LT and ST), EAEC, *C. difficile, Entamoeba, Giardia, Cryptosporidium, Aeromonas, Plesiomonas,* and *Providencia.*

Genetic studies started with extraction of DNA from blood, and analysis for single nucleotide polymorphisms by PCR-pyrosequencing or direct sequencing. Fecal cytokines were measured by ELISA, and clinical outcomes and associations determined by the $\chi 2$ test. To date >1,000 subjects have been enrolled, and 698 completed the study with 6 month follow-up. Females constituted 55% , white 74%, Hispanic 20%, Asian 2%, African American 2%, and Native American <1 %. The mean length of stay was 31d (7-45), and travelers' diarrhea was seen in 64% by diary review. Of those, 41% sought attention. The mean onset was 9 days (1-31), the time ill before being seen was 39 h (2.5 -141), and the number of unformed stools was 5.7 (2-17).

Toll-Like Receptors were represented in the results of the SNPs analysis. TLRs are evolutionarily conserved receptors that play a key role in the induction of immunity and inflammation. At least 11 TLRs have been identified, a picture complicated by their ability to function in complexes. The TLRs are relatively specific for bacterial, viral, and parasitic products, for example, TLR4 is a receptor for LPS, and TLR5 is specific for flagellin. TLR 5 is present in intestinal epithelial cells, dendritic cells, microvascular endothelial cells and monocytes. Basolateral enterocyte activation of TLR5 leads to phosphorylation and nuclear translocation of the transcription factor NFκB. Two TLR5 SNPs were studied, 1174 C →T and 1775 A →G. SNP 1174, which was significantly associated with increased diarrhea ($p=0.04$), results in $TLR5^{392STOP}$ with a loss of function and has previously been associated with hypo-responsiveness and mortality due to *Legionella.*

The study of fecal chemokines showed that of IL-1 β, IL1r α, TNFa, IL-6, IL-8, and INF γ , IL-8 was the most highly induced. Of *Shigella,* EAEC, ETEC, and *Salmonella, Shigella* was the most inductive. Interleukin-8 is a chemokine that attracts neutrophils to sites of inflammation and activates macrophages; elevated fecal levels occur in response to infection during bacterial diarrhea and from enterocyte cell lines exposed to these pathogens. Significantly increased IL-8 production has been described in response to AggR alone and combinations of virulence determinants. A polymorphic allele (-251A) of IL-8 has been implicated to a trend toward increased response of PMN to LPS. In addition, the -251A allele increases susceptibility and severity of RSV bronchiolitis, increases susceptibility to TB, and decreases susceptibility to colon cancer. The IL-8 -251 T→A SNP is significant in travelers' diarrhea, however fecal levels of IL-8 vary greatly according to genotype from ~135 pg/ml (AA), to ~20 pg/ml (AT) and 0.5 pg/ml (TT). Intriguingly, 44% of the AA travelers, 66% of the AT, but 0% of the TT study travelers who visited clinics during their stay had EAEC identified from stool culture, suggesting a significant protective effect of the TT genotype against EAEC.

The immunoregulatory cytokine Interleukin-10 is anti-inflammatory, leading to decreases IL-1, TNF-α, IL-8, and increased levels of IL-1RA, TNFsr. In addition, IL-10 stimulates NK cells, enhances production of IL-4 and IgA. IL-10 SNPs in the promoter region have been associated with sepsis, coronary artery disease, increased inflammation in *H. pylori* infection, susceptibility to HIV infection, psoriasis, and IBD. The -592 IL–10 SNP only appeared to be significantly associated with cryptosporidiosis among the enteropathogens tested, with a significantly greater number of cases occurring among the CA genotype compared to the AA or CC homozygotes. Overall, the non-CC individuals were more likely to suffer from diarrhea but less likely to present at the clinic. The most significant protection was seen against cryptosporidiosis, with the CC genotypes protected ($p=0.001$) relative to non-CC genotypes. The SNPs in IL-8 and IL-10 appear to interact, leading overall to major host-dependent response and vulnerability to travelers' diarrhea.

RESAERCH NEEDS:

- Genetic polymorphism determination in diarrhea studies conducted among pediatric and other populations both in developed and developing countries The significance extends to both acute and chronic infections caused by enteric pathogens, including the opportunistic infections occurring in the HIV infected

- Genetic polymorphism determination in vaccine safety and efficacy studies. These data may be informative regarding reactogenicity, intussusception and vaccine failure among diverse populations in different geographical settings, and may facilitate the determination of optimal schedules
- Determine the possible role of host genetics on a range of post-diarrhea complications such as Guillian-Barre Syndrome, reactive arthritis, persistent diarrhea, malnutrition and irritable bowel syndrome

Small molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization

Deborah T. Hung, M.D., Ph.D.

Vibrio cholerae, the etiological agent of cholera, exhibits coordinate genetic regulation of several virulence determinants, most significantly the potent cholera toxin (CT), and the essential intestinal colonization factor, the toxin coregulated pilus (TCP). The regulatory pathways that influence expression of virulence genes in *V. cholerae* have been subject to detailed research in recent years, including the identification of ToxT as a key transcriptional activator of CT and TCP. A high throughput screen was developed based on a *ctx-tetA* reporter construction to identify small molecule inhibitors of ToxT within the 50,000 compound library of Chembridge. Two rounds of analysis led to 109 (0.22%) compounds which inhibited the growth of the *ctx-tetA* construction in the presence of tetracycline. Of these 109 compounds, 15 were selected based on their structure, effect on bacterial growth and inhibition of CT production. The lead compound, named Virstatin, {4-[N-(1,8-naphthalimide)]-n-butyric acid} had no effect on the growth of classical biotype strain O395 (600uM) or El Tor biotype C6706 (1200uM). The inhibition of CT and TcpA expression was studied under *in vitro* growth conditions that stimulate toxin and pilus expression. Minimum inhibitory concentrations of 3uM (for strain O395) and 40uM (for C6706) were determined to reduce toxin production to undetectable levels.

Activity of Virstatin

Virstatin was determined to inhibit ToxT by several methods including demonstration that virstatin inhibits ctx transcription, but not toxT transcription. Additionally, virstatin inhibited the activity of ToxT expressed under a heterologous promoter (pBADtoxT complementation of O395Δ*toxT*), and also prevented ToxT activation of ctx-lacZ cloned in *E. coli.*

An escape mutation of ToxT was identified by screening a mutated pBAD24-*toxT* library, allowing identification of a L113P mutation within the N-terminal domain of ToxT which resulted in relative resistance to virstatin. In comparison to the wild-type ToxT which was inhibited by 90% at 10ug/ml, the escape mutant (C6706mut) was unaffected by virstatin at 50ug/ml, and showed 80% activity at 60ug/ml.

The effect of virstatin was evaluated using the fully virulent El Tor cholera strain C6706 in the infant mice colonization model. Virstatin (50ug) was delivered with cholera inoculation with or without a small concentrated boost at 3.5-4 hours, comparing strain C6706wt to S533, a non-O1, non-O139 CT-negative strain that lacks the TCP island, and hence is both negative for *tcpA* and *toxT.* Experiments using virstatin at 500ug/ml (which is not toxic *in vitro*), showed that drug applied at inoculation and boosted at 3.5-4 h reduced recovery of bacteria by 4-logs, an effect that did not occur with control escape mutant C6706$_{mut}$.

Finally, virstatin decreased C6706 colonization in infant mice by 3 logs at 36 and 60 hours evern when administered 12h after inoculation of the mice with bacteria, suggesting that in established infection, virstatin could have utility in clearing infection even after disease is diagnosed.

Activity of alrestatin

We have synthesized and tested 30 structural analogues of virstatin and have identified an analogue {4-[N-(1,8-naphthalimide)]-n-ethanoic acid}, alrestatin, which demonstrates similar potency as virstatin in vitro, with an MIC of 4uM, for inhibition of ToxT. In the infant mouse model of infection, alrestatin was similarly active both in early and late inoculation, though higher doses were required to obtain the same in vivo efficacy, requiring 100ug/mL dosing in the infant mouse. When extrapolated to a 60 kg person, this would translate to 1 gm po tid of alrestatin compared to 500 mg po tid for virstatin.

Alrestatin was developed by Ayerst as an aldose reductause inhibitor for diabetic neuropathy. It was administered to diabetics at 1-1000 mg/kg/day, with an LD50 of 2500 mg/kg in rats. In addition, diabetics were treated up to 4 months, with the major toxicity being phototoxicity. It is no longer in development due to short serum half-life. However, we are currently pursuing the possibility of obtaining GMP material from Ayerst for testing in *V. cholerae* challenge studies.

In conclusion, a high-throughput phenotypic screen can be used to identify small molecule virulence inhibitors that exhibit *in vivo* efficacy against bacterial infection in the absence of any chemical or structural target information. A new class of highly targeted, small molecule inhibitors of virulence may provide novel approaches to circumvent traditional routes to antimicrobial resistance, and offer a therapeutic option that minimizes the perturbation of intestinal flora. Treatment of infections with antivirulence drugs may limit the risk of treatment failure, and will not engender widespread resistance to conventional antibiotics. Small molecules that inhibit virulence gene expression may synergize with traditional drugs to help clear infection. Members of this new class of drugs can be defined as '*A synthetic or natural compound that inhibits the expression of microbial virulence factors in vivo.'*

Novel Therapeutics for HUS
Tom Obrig, Ph.D.

The progression of disease after the ingestion of food contaminated with enterohemorrhagic *E. coli* (EHEC) becomes apparent with the onset of diarrhea, abdominal pain, and vomiting approximately three days post-infection. Two days later, diarrhea becomes bloody in 90% of cases. After 4 to 5 days of enterohemorrhagic colitis, the diarrhea resolves spontaneously in 85-90% of cases, but progresses to acute renal failure and the hemolytic uremic syndrome (HUS) in 10-15% of cases. The timeline of disease progression indicates a window of opportunity to intervene during the systemic toxemia phase of disease from day 2.5 to day 7 post-infection, when the bacterial Shiga toxin (Stx2) and lipopolysaccharide (LPS) drive the transition to bloody diarrhea and the risk of HUS.

The observed pathology during the window of opportunity includes host inflammation and coagulation/thrombosis which become possible therapeutic targets in control of the host response. Another potential target is the blocking of toxin receptors. Employment of a combination of these approaches is also possible.

The primary virulence determinants of EHEC impact host cells by different mechanisms. The potent Stx2 binds selectively to glycosphingolipid (Gb3) expressed on the surface of host cells and activates the stress response, inhibits protein synthesis, induces cytokines, and initiates apoptosis. LPS binds TLR4, induces cytokines/chemokines, and magnifies the Stx2 response by inducing the expression of additional Gb3 on target cells.

Adenosine is an anti-inflammatory compound of interest in these studies. Adenosine is normally produced by damaged host cells in many situations. However, newer synthetic analogs of adenosine may offer pharmacological benefits. Adenosine works by binding to and activating specific G protein-coupled receptors on certain cell types to activate a cyclase that generates cyclic AMP (cAMP). Thus, adenosine is a high affinity agonist of the adenosine A_{2A} receptor for cAMP production and cAMP indirectly blocks adhesion of monocytes to the vascular endothelium as part of its anti-inflammatory function.

The study of anti-inflammatory compounds necessitated the development of a mouse model of kidney inflammation. Injection of mice with a lethal dose of Stx2 (200 ng/kg), with and without a sublethal dose of LPS (300 ug/kg), was followed temporally by euthanasia, removal of kidneys, and analysis of inflammatory cells, cytokines/chemokines, and selectins/integrins. Renal mRNA was analyzed by microarray, RNase protection assays, and real-time rt-PCR, while renal protein was analyzed by ELISA, western blot and immunohistochemistry. The following temporal events were observed. When Stx2 was applied alone, serum creatinine levels increased steadily reaching a three-fold increase over 84 hours. LPS alone induced Gb3 on renal vascular endothelium. When both LPS and Stx2 were employed, a unique series of events occurred that included induction of renal chemokines and cytokines that attracted monocytes and neutrophils into the kidney, leading to increased adherence of inflammatory cells to endothelia, the activation of platelets, coagulation, thrombosis, endothelial damage, and other manifestations of HUS analogous to those seen in patients. Monocyte/macrophage recruitment to the kidney medulla and cortex of Stx2/LPS-treated mice showed a steady 5 to 10-fold increase over the course of 72 hours. The chemokines for monocyte chemotaxis in mice include MIP-1α / CCL3, MCP-1 / JE / CCL2, and RANTES / CCL5, which can be identified using immunostaining of tissue sections, revealing intense expression and vascular damage surrounding monocytes when Stx2 and LPS are co-administered.

The ability of the adenosine A_{2A} agonist ATL to inhibit the early events in the Stx2/LPS response was assessed using ELISA assay for renal MIP-1α, MCP-1/JE, RANTES, and monocyte/macrophage recruitment, in the presence and absence of ATL146e at 10ug/kg every 6 hours. Significant differences were detected in the induction of all markers, and the peak cytokine/chemokine responses seen at 2-12 hours were reduced to approximately 60%. In addition, macrophage/monocyte recruitment into the kidney was reduced from a maximum of ~55 cells per field of view to ~12 at 72 hours. Similarly, the effect of ATL146e on renal TNF-α was significant, with treated animals showing significantly reduced expression until 72 hours, when levels returned to baseline in treated and control animals, a pattern also seen in renal IL-1β measurements.

Platelets are activated in HUS due, in part, to the action of Stx2 on the endothelium, which results in the expression and release of chemokines such as SDF-1α and SDF-1β that are co-activators of platelets. The effects of Stx2, LPS, and ATL-146e on the release of SDF-1α and SDF-1β were measured using human microvascular endothelial cells (HMEC). Platelet aggregation in vitro is rapidly activated over a period of 5 seconds. In the mouse model, ATL146e reduces the Stx2-dependent peak renal platelet count by 90%. The mechanism of ATL146e action, therefore, appears be through inhibition of chemokine induction at the endothelial level, and by inhibiting the release of cytokines by monocytes in the kidney.

The effect of another longer acting adenosine A_{2A} agonist, ATL303 administered i.p. every 12 hours for 7 days, was tested in a series of preliminary experiments. When ATL303 was administered to Stx2 /LPS treated mice, the time to death was extended in a dose-dependent manner from 2 days (10ug/kg) to >12 days (20ug/kg).

In conclusion:

- A_{2A} Receptor agonists inhibit LPS/Stx2-induction of renal chemokines for monocyte migration into kidney of mice.
- A_{2A} Receptor agonists inhibit Stx2-induction of renal cytokines produced by monocytes.
- A_{2A} Receptor agonists inhibit Stx2-induced accumulation of monocytes and platelets in kidneys of mice.
- A_{2A} Receptor agonists reduce Stx2-induced lethality in mice.

RESEARCH NEEDS:

- Identification of lead therapeutic candidates for preclinical and clinical development.

MarA Inhibitors
Michael Alekshun

During the process of infection a pathogen must first invade a host and then attach itself to a tissue. Once inside the host a pathogen may express toxins or other host avoidance proteins and is under constant pressure to respond to changes in pH, temperature, osmolarity, and the presence of antibiotics. These events are primarily controlled at the transcriptional level by regulatory elements such as the multiple adaptational response (Mar) system of *E. coli*. The Mar system is a regulatory locus that encodes a transcription activator, termed MarA, which is a member of the AraC family of DNA binding proteins. Proteins within this highly conserved family are characterized by a dual HTH DNA-binding motif. There are two subfamilies of AraC proteins, the small (~15kDa) group such as MarA and SoxS which only possess a DNA binding domain, and the larger proteins (~30kDa) such as AraC, Rob, ToxT, BfpT, and ExsA that possess the DNA binding domain and an additional domain with an another function. Over 1,000 orthologs of AraC proteins have been identified in gram-negative pathogens such as *E. coli* (UPEC and EPEC), *Shigella, Salmonella, Yersinia, Proteus, P. aeruginosa, Enterobacter, Klebsiella, V. cholerae, N, gonorrhea,* gram-positive organisms, including *S. aureus, Streptococcus*, *Enterococcus, Bacillus*, and the mycobacteria.

Experiments in a murine pyelonephritis model of infection using a multidrug resistant *E. coli* strain illustrate the importance of MarA, SoxS, and Rob in virulence. Infection with the wild type organism persisted for at least 11 days. However, when *marA, soxS,* and *rob* were deleted, the infection was cleared within 5 days. Bieber, et al reported that in EPEC, deletions in PerA severely attenuated infection in humans (Science 1998, **280:**2114). In *V. cholerae,* ToxT plays a significant role in the infant mouse model, with mutants of either El Tor or classical biotype strains showing significantly decreased lethality at 24 hours (Champion et al, 1997 Mol Micro **23:**223). LcrF, a MarA analog from Yersinia, controls expression of a type III secretion system, which injects cytotoxic Yops into macrophages and other host cells. Some Yops, like YopJ inhibit the MAPK cascade while others including YopE disrupt actin in microfilament formation and block phagocytosis and inhibit the oxidative burst like YopH.

The function of MarA is to regulate a number of cellular functions including multi-drug resistance, metabolism, DNA repair, biofilm formation, cell envelope synthesis, tolerance of organic solvents, virulence, and transport. The crystal structure of MarA has been solved (Rhee et al PNAS**95:**10413 and Kwon et al 2000 Nature Struct Biol. **7:**424.) and the interactions of MarA and Rob with DNA have been defined.

Many small-molecular weight MarA inhibitors have been identified. These agents inhibit protein-DNA interactions in vitro and have shown activity against whole bacterial cells in virulence assays. Multiple compounds have been tested against *P. aeruginosa* (ExsA), *Y. pseudotuberculosis* (LcrF), and *E. coli* (SoxS). Several exhibit efficacy in an E. coli pyelonephritis and Yersinia lung infection models. In the former, mean bacterial counts in the kidney were reduced by over 3.5 logs when an exemplary compound was administered at 1mg/kg over a period of five days. Dose escalation studies with this agent showed a significant (4-log) reduction in bacteria recovered from the kidney.

Future plans for developing MarA inhibitors will exploit a non-antibacterial approach for infectious disease therapy that will hopefully avoid the traditional drivers for the development of antibacterial resistance. Since MarA and its relatives are well-conserved targets, the opportunity for developing therapy against a variety of agents, particularly *Yersinia, E. coli*, Pseudomonas, and *Vibrio*, is envisioned. Presently, efficacy in vivo has been shown for *E. coli* and *Yersinia,* and future studies are expected in order to develop screening systems for other agents.

The research needs identified by these studies include the full range of preclinical development and clinical support. Large pharmaceutical companies have deserted this exciting field, just as new opportunities are emerging.

Rifaximin for the Treatment and Prevention of Enteric Infection
Robin McKenzie

Diarrheal diseases are the second leading cause of morbidity and mortality worldwide, causing over 3 million deaths per year, predominantly in young children in developing countries. Among travelers, diarrhea is the most common illness: there are approximately 10 million episodes of travelers' diarrhea each year. Bacteria account for 33-50% of episodes of pediatric diarrhea in developing countries and about 80% of travelers' diarrhea. The most common enteropathogen identified among travelers is *E. coli*.

Rifaximin, a broad-spectrum rifamycin, inhibits RNA synthesis. It is minimally absorbed (<0.4%) and well tolerated. Even when the colon is inflamed, absorption is minimal. Given to volunteers in a *Shigella* challenge study, rifaximin produced peak plasma concentrations and areas under the concentration-time curve that remained similar from day one to day three. Rifaximin has a relatively minor impact on intestinal coliforms, reducing fecal coliforms by about one log. In countries where it has been used for several years, no stable resistance has been reported.

Four field trials evaluated rifaximin as treatment for travelers' diarrhea. Studies conducted in Mexico (72 subjects), Mexico and Jamaica (187 subjects), and Mexico, Kenya, and Guatemala (380 subjects) compared rifaximin to trimethoprim-sulfamethoxazole, ciprofloxacin, and placebo for treatment of travelers' diarrhea. A 3-day course of rifaximin (400 mg twice daily) was comparable to ciprofloxacin in terms of duration of diarrhea after treatment initiation (median, 26 hours vs. 25 hours), and cure rates (87% vs. 88%). Compared to placebo, a 3-day course of rifaximin (200 or 400 mg three times daily) significantly reduced the duration of diarrhea (33 hours for each of the rifaximin groups vs. 60 hours for placebo).

In a fourth (unpublished) randomized, double-blind study, 399 adult travelers to Mexico, Guatemala, India and Peru who developed diarrhea were treated with rifaximin (200 mg three times daily), ciprofloxacin, or placebo. Again the time after treatment initiation until the last unformed stool was comparable for rifaximin (32 hours) and ciprofloxacin (29 hours) and significantly less than for placebo (66 hours). However, subjects with invasive pathogens, (*Campylobacter, Shigella,* and *Salmonella*) and those with fever and/or blood in their stools did not respond well to rifaximin probably because invasive bacteria are not susceptible to treatment with a luminal agent.

Since rifaximin is not absorbed but achieves very high luminal concentrations, it is attractive as a potential prophylactic agent for travelers' diarrhea. In a placebo-controlled, double-blind study 210 adults arriving in Mexico were randomized to one of four groups to receive rifaximin (200 mg once, twice or three times daily) or placebo. During the two weeks of the study, 54 % of controls and 15% of rifaximin recipients developed travelers' diarrhea (protective efficacy, 72%). There was no difference in the diarrhea rates for the three different rifaximin doses.

While travelers' diarrhea in Mexico is largely caused by *E. coli*, in some other countries invasive organisms, such as *Shigella* and *Campylobacter*, occur more commonly. Even though rifaximin is not effective for treatment of shigellosis, it may prevent shigellosis by killing the bacteria in the gut lumen before they invade the mucosal epithelium. In a randomized, double-blind trial 15 subjects received rifaximin (200 mg three times daily for 5 days) and 10 received placebo. All were challenged with 1000-1500 cfu of *S. flexneri* 2a. Sixty % of controls but none of the rifaximin-treated subjects developed shigellosis. Furthermore, no one in the rifaximin group became colonized or demonstrated an immune response to the *Shigella* strain, in contrast to the control group, among whom 50% shed the challenge strain, and 80% developed an immune response (serum IgA or IgG or IgA antibody secreting cells).

In conclusion, for treatment of travelers' diarrhea, rifaximin was comparable to ciprofloxacin in efficacy and was most effective for cases caused by *E. coli* and cases for which no pathogen was identified. Rifaximin should not be used for treatment of travelers with fever or blood in their stools since these individuals may have an invasive infection not accessible to a luminal agent. Used for prophylaxis, rifaximin reduced the incidence of travelers' diarrhea from 54% to 15% during a two-week trial in Mexico (72% efficacy). Furthermore, it prevented shigellosis following challenge, probably by eradicating the organisms before invasion. In these studies adverse events were similar to those seen with placebo.

Many questions are raised by these studies. What is the role of rifaximin in treating diarrheal diseases in adult travelers and in children? Would a shorter course (single dose) be effective? Will rifaximin be efficacious for treating enterohemorrhagic *E. coli* and *Clostridium difficile*? What is its role for prophylaxis, and what dose should be used? Will prophylaxis of travelers' diarrhea prevent irritable bowel syndrome? Will resistance to this drug become a problem?

Nitazoxanide
Paul Hoffman

Nitazoxanide is a broad-spectrum drug manufactured by Romark Laboratories marketed under the trade name of ‘Alinia’. The activity of nitazoxanide has been reported to go far beyond the FDA-approved treatment of cryptosporidiosis and giardiasis. The USDA has approved the drug for *Sarcocystis neurona* and off-label uses include treatment for helminth infection, *Trichomonas, Entamoeba, Clostridium difficile,* and viruses such as hepatitis C.

Nitazoxanide is a structural analog of thiamine pyrophosphate, and as such acts as an inhibitor of pyruvate:ferrredoxin oxidoreductase (PFOR) that converts pyruvate and CoA to acetyl CoA. Humans and many bacteria (such as *E. coli*) utilize pyruvate dehydrogenase (PDH) for this reaction, and therefore are not inhibited by nitazoxanide. Nitazoxanide shows high levels of activity against both metronidazole-sensitive and resistant *Trichomonas vaginalis* , with the greatest activity *in vitro* being observed when organisms are cultured under anaerobic conditions where PFOR plays a leading role in metabolism. Studies on the electron transfer activity of PFOR *in vitro* in the presence of nitazoxanide showed reduction in electron transfer in *T. vaginalis, E. histolytica, G. intestinalis, C. difficile, C. perfringens,* and *H. pylori.* The inhibitory action of nitazoxanide is through reversing the binding of pyruvate to enzyme-bound thiamine pyrophosphate (TPP), of which it is a structural analog. The bioactivity of nitazoxanide is pH dependent, and the protonated form is inactive. Despite widespread clinical use, no resistance has emerged to nitazoxanide, although resistance is theoretically possible through mutations in PFOR that maintain activity and TPP binding, but not binding by nitazoxanide. Nitazoxanide may be the first of a new class of antimicrobial drugs that inhibit function of a cofactor, rather than by inhibiting enzyme action.

Remaining research questions include establishing the molecular mechanism by which nitazoxamide inhibits helminths, since these parasites lack PFOR. The activity against viruses, if it is real, also remains unexplained. In addition, the experience with nitazoxamide provides an experimental drug discovery system that might be particularly appropriate for the development of therapeutics for use with intestinal pathogens, since activity within the lumen and minimal intestinal uptake would be advantageous from a toxicity standpoint.

Probiotics for Gastrointestinal Infections
Richard A. Oberhelman, M.D.

As probiotics becomes increasingly common in medical literature, pediatricians may be facing questions from parents about their use in children.

These dietary supplements are living microorganisms that change the microbial flora of the gut or another mucosal surface. Within the past 15 years, scientists have begun to study specific probiotic organisms in earnest. While many health benefits have been ascribed to probiotics, the most convincing are in the treatment of certain types of infectious diarrhea. We have recently reviewed the use of probiotics in the management of infectious diseases (Alvarez M, Oberhelman RA. Probiotic agents and infectious diseases: A modern perspective on a traditional therapy. Clin Infect Dis 32:1567-76, 2001).

The most commonly used probiotic organisms are certain strains of *Lactobacillus*; *Saccharomyces boulardii*, a nonpathogenic yeast related to (but not the same as) baker's yeast; and *Bifidobacterium*, a genus of anaerobic gram-positive rods that can constitute up to 99% of the fecal flora in exclusively breast-fed infants.

Probiotics are different from prebiotics, a new term used for non-digestible food ingredients that promote the growth of beneficial bacteria in the gastrointestinal tract. Prebiotics usually are complex oligosaccharides, and they are sold commercially (primarily in Europe and Japan) as food supplements for infants and adults.

Recent studies demonstrate that not all *Lactobacillus* strains are effective probiotics. Most probiotics studied are selected for characteristics such as ability to colonize and adhere to the gut mucosa, production of antimicrobial substances, and ability to stimulate mucosal immunity *in vitro.*

The most widely studied probiotic organism is *Lactobacillus casei* (or *L. rhamnosus*) strain GG (LGG), although other strains of *L. reuteri* and *L. acidophilus* also have been studied and found to be effective in some clinical trials. Interestingly, lactose fermentation by probiotic organisms to aid in digestion of sugars does not appear to be a major mechanism of action, since many of the most effective probiotic agents are poor lactose fermenters.

Promising clinical trial results

Controlled clinical trials from many countries have shown that LGG and similar probiotics shorten the duration of watery diarrhea, especially watery diarrhea due to rotavirus. The greatest benefit has been shown in studies from northern European countries in which 60% to 90% of cases were associated with rotavirus.

In developing countries where rotavirus accounts for a much smaller proportion of cases and where bacterial and parasitic agents are more common, probiotics only demonstrate a benefit in the subgroups with watery diarrhea (i.e., no benefit from probiotic treatment was seen in cases of invasive or bloody diarrhea). While most clinical trials only have included hospitalized inpatients with diarrhea, a recent Danish study (V Rosenfeldt *et. al.*, Pediatr Infect Dis 21:417-9, 2002) also demonstrated benefit from LGG treatment in outpatients with milder diarrhea recruited from a child care center.

Several smaller studies also have shown a benefit from LGG and *S. boulardii* treatment of antibiotic-associated diarrhea and relapsing diarrhea due to *Clostridium difficile*, but data do not suggest that probiotics are effective against specific pathogens other than rotavirus and *C. difficile.*

A recent meta-analysis pooled the results of eight placebo-controlled trials, including 731 children with diarrhea who took a variety of probiotic agents (Szajewska H and Mrukowicz JZ. *J Pediatr Gastroenterol Nutr.* 2001;33:S17-S25). The pooled relative risk of diarrhea in the probiotic group was 0.43. These studies mostly were of rotaviral diarrhea, and LGG was the only probiotic agent with sufficient data to demonstrate a consistent effect on reduction of diarrhea in this analysis.

Studies of probiotics for treatment of traveler's diarrhea in adults and for prevention of diarrhea in children have shown modest benefits in some cases, but it is difficult to make generalizations about these potential uses because of the limited number of studies and differences in the study populations. In a randomized placebo controlled trial in 204 undernourished children receiving LGG or PL in liquid gelatin 6 days a week for 15 months, there were significantly fewer diarrheal episodes the in LGG group (5.2 vs. 6.0 episodes/child/yr. [e/c/y], p=0.028). The greatest difference was seen in 18-29 month old group (p=0.004) but the effect was primarily limited to non-breastfed children (BF: 6.6 e/c/y LGG vs. 6.3 e/c/y PL, N.S.; NBF: 4.7 e/c/y LGG vs. 5.9 e/c/y PL, p=0.005) [Oberhelman et. al.; J Pediatr 1999; 134: 15-20].

A small number of studies also have shown some benefit from treatment with several *Lactobacillus* strains in women with recurrent *Candida* vaginitis. Future applications under investigation are treatment of bacterial vaginosis, mucosal vaccine development, prevention of HIV and STD transmission in women, control of inflammation and inflammatory bowel disease, and treatment of multidrug-resistant organisms on body surfaces.

Probiotics in pediatrics

What do these studies mean for pediatricians practicing in the United States? First, it is important to educate parents that these studies were done with scientifically selected strains, so one would not expect to see the same benefit from commercially produced yogurt from the grocery store.

Scientifically tested probiotic agents marketed commercially in the United States include LGG (Culturelle; CAG Functional Foods) and *S. boulardii* (Florastor; Biocodex Inc.). Many probiotic products are sold on the Internet by various vendors, but quality control of these products is variable. The probiotic strains studied are more widely available in Europe and the Far East, where they are marketed in a variety of ways (e.g., as food supplements for infant formula, commercial yogurt and lyophilized powder capsules). Probiotics usually are distributed over-the-counter and dosing is frequently not well standardized.

Second, probiotic organisms are generally recognized as benign and are thought to be safe, although as food supplements they have not been subjected to the rigorous safety studies and manufacturing standards required for drugs. Independent microbiologic analyses have not always identified the ingredients listed on the label, and other quality control problems reported (mostly in products made outside the United States) are inconsistent numbers of microorganisms and presence of potential pathogens such as *Enterococcus faecium.* While there are several reports of opportunistic infections with *Lactobacilli* in the medical literature (mostly in patients with underlying diseases), there have been no increases in cases of *Lactobacillus* bacteremia in countries like Finland where probiotic use and surveillance for probiotic-associated infection are common. Only a handful of studies have evaluated the safety and efficacy of probiotics in HIV-infected individuals. Although these have not demonstrated adverse effects in a small number of HIV-positive persons treated with *Lactobacillus reuteri* and *Saccharomyces boulardii*, more data will be needed before wider use in this population can be promoted.

There are data to support the use of effective probiotics for watery-diarrhea, especially if it is known to be rotaviral, and perhaps in some cases of antibiotic-associated diarrhea or relapsing *C. difficile* colitis. However, we do not have sufficient data to clearly define the role that these products should play in general pediatric practice. Further studies to evaluate the efficacy of probiotics in outpatients are needed. With time probiotics likely will be more readily available, and physicians and patients should learn how these products can be used most effectively.

Future research needs in the area of probiotics in diarrheal diseases include:
•Studies of the effects of repetitive probiotic therapy on long term growth and development. To date most studies have focused on the effect on single diarrheal episodes, which does not allow detection of long term effects on nutritional status that may be significant.

•Further studies of safety issues

•Better understanding of the physiology of probiotic organisms and mechanisms of protection

•Better understanding of the role of malnutrition, lactose intolerace, and breastfeeding as cofactors that impact probiotic efficacy

Human Milk Glycans
David S. Newburg

The study by Grulee and colleagues of over 20,000 infants of 0-9 mo. age in Chicago from 1924-9 provided compelling evidence of the protective efficacy of human milk compared to either full or partial feeding with human milk substitutes. Among the data were striking mortality figures, showing ten times the rate of deaths among the artificially fed group than among the breast-fed infants. Compared with breastfeeding, the Grulee study indicates that artificial feeding confers an increased relative risk for morbidity of 3.1 fold and mortality of 7.1 fold. Additional benefits supplied by ideal nutrition extend beyond the immediate prevention of acute infectious disease.

Protective components of human colostrum and milk include prebiotic elements, sIgA, and multifunctional agents of the innate immune system. In addition, the ability of the oligosaccharides and glycoconjugates of human milk to protect the infant is now becoming more evident. This protection may be some of the strongest offered by human milk.

The many hundreds of distinct human milk oligosaccharides have been characterized according to weight using MALDI-MS, and several of the smaller macromolecules have been characterized. Lacto-N-fucopentaose I, II, and III are comprised of Gal, Fuc, GlcNAc, Gal, Glc with the isomers distinguished by the linkage between the fucose residue to the terminal galactose or penultimate GlcNAc, which may be through the second (to galactose), third or fourth (to GlcNAc) carbon. Glycoconjugates may protect the intestinal epithelium in a variety of ways. They may prevent the early events in pathogen colonization, or competitively inhibit binding of toxins such as the *E. coli* stable toxin (ST).

Oligosaccharides containing α1,2-linked fucose can inhibit ST of E.coli, are present in milk at a concentration of ~30 ppb, and can strongly inhibit ST at 30 ppb. Furthermore, glycans containing α1,2-linked fucose strongly bind to *Campylobacter jejuni*. In a study of diarrhea among nursing Mexican children, the presence of specific Lewis-ABO(H) blood group antigens in milk correlated with significant protection against diarrhea; again, oligosaccharides containing α1,2-linked fucose were protective. A likely mechanism is the inhibition of pathogen-driven processes of bacterial attachment and invasion. Experimental systems utilizing transgenic mice with human α1,2-linked fucose activity in their mammary glands showed that in infant mice consuming transgenic milk containing α1,2-linked fucose, intestinal colonization declined to 0%, while those nursing non-transgenic dams were unable to eliminate the infection. Additional experiments suggest that fucosylated glycans inhibit Norwalk virus and other human caliciviruses.

Human milk oligosaccharides seemed to protect nursing infants against diarrhea caused by ST-producing *E. coli* strains; the levels of 2-linked fucosyloligosaccharides were significantly lower in the milks consumed by symptomatic children than in milk consumed by asymptomatic and uninfected children. Low levels of 2’FL (H-2 epitope) in milk were also associated with approximately four fold higher risk of Campylobacter-mediated diarrhea; high levels of 2’FL(H-2) correlated with heightened protection.

High levels of LDFH-1 (Le^b) in milk were associated with significantly reduced incidence of norovirus diarrhea, and diarrhea from all causes was significantly lower in groups with high levels of 2-linked oligosaccharides in milk.

There is evidence for inhibitory activity by human milk glycoconjugates against *Streptococcus pneumoniae*, enteropathogenic *E. coli* (EPEC), *Campylobacter jejuni*, and Stable toxin of *E. coli* (ST). Glycopeptides appear to protect against enterohemorrhagic E. coli (EHEC), glycoprotein against rotavirus, glycosaminoglycan against Human Immunodeficiency Virus (HIV), and mucin against S-fimbriated *E. coli*.

Glycolipids associated with toxin binding are as follows: GM1 (cholera toxin, labile toxin, and the toxin of *C. jejuni*) and Gb3 (shiga toxin I and II from Shigella or *E. coli*). Sulfatide inhibits HIV and *Salmonella*-induced pathogenesis.

The human milk glycoconjugates may act as cell surface or toxin receptor homologs that absorb toxins or subvert pathogen-directed attachment and invasion. For example, expression of soluble competitors of pathogen targets may compete with epithelial binding sites, allowing unattached pathogens or toxins to be removed by peristalsis.

Follow-up research should include more detailed characterization of the inventory and activity of the high molecular weight glycans of human milk, develop methods for large scale production, and test specific glycoconjugates in a variety of systems against *Campylobacter* spp, cholera, ST, caliciviruses, and EPEC.

PHAGE THERAPY

Alexander Sulakvelidze, Ph.D.

Bacteriophages - viruses that kill bacteria - were first identified in the early part of the 20th century by Frederick Twort and Felix d'Herelle who called them *bacteriophages* or bacteria-eaters (from the Greek *phago* meaning *to eat* or *to devour*). Bacteriophages (or 'phages' for short) are ubiquitous, obligate parasites highly specific for their bacterial host. Because of their remarkable antibacterial activity, phages were used to treat diseases of humans and agriculturally-important animals almost immediately after their discovery. The first reported application of phages to treat infectious diseases of humans was by Bruynoghe and Maisin in 1921, who successfully used bacteriophages to treat staphylococcal skin disease. In the 1930-1940s, *Eli Lily and Co*. manufactured several therapeutic phage products. Other major companies involved in therapeutic phage production included *E.R. Squibb and Sons* and *Swan-Myers* (Abbot Laboratories). However, with the advent of antibiotics, the initially strong interest in phage therapy declined in the West. Overseas activity survived longer. The Russian and German armies routinely used phage preparations, and from the 1920s to the current day, phage therapy has been utilized in Eastern Europe and the former Soviet Union.

Phages have been used against cholera in the historic "Cholera Study" in India. *Shigella* phages have been correlated with the decreased incidence of dysentery. Numerous additional publications – most of them published in non-English scientific literature – report on various applications of bacteriophages in clinical settings. Some of the commercial phage products from the Laboratoire du Bactériophage in France included *Bacté-coli-phage, Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage*, and *Bacté-staphy-phage*, Eli Lily has *Colo-lysate, Ento-lysate, Neiso-lysate,* and *Staphylo-lysate*, *Colo-jel, Ento-jel,* and *Staphylo-jel*. Currently, ImBio in Russia is producing phage-based preparations in liquid, tablet, and cream formulations for treating bacterial dysentery (**"**Bacteriophagum dysentericum polyvalentum in tabulettis"), the early stages of salmonellosis ("Bacteriophagum salmonellae gr.ABCDE liquidum et siccum cum indumento acidoresistentis"), general gastrointestinal disorders ("Bacteriophagum coliproteicum liquidum"), *S. aureus* infections ("Bacteriophagum staphylococcus**"**), and *P. aeruginosa* infections ("Bacteriophagum *Pseudomonas aeruginosa* liquidum"). Another company in Russia, Biophag, currently manufactures at least two complex phage preparations ("Bacteriophagum" and "Piobacteriaphagum") targeting various bacterial pathogens. CMBP in Georgia produces *PhagoBioDerm*, and Eliava Institute of Bacteriophage sells several therapeutic phage preparations, including *IntestiPhage* and *PyoPhage*.

From a clinical standpoint, phages are very safe. This is not surprising, given that humans are exposed to phages from birth (and, possibly, even *in utero*). Indeed, bacteriophages are arguably the most ubiquitous organisms on earth. In the USA, approximately 3×10^9 coliphages are shed per person per day, which extrapolates to approximately 780,000,000,000,000,000 coliphages shed daily in this country alone. One ml of non-polluted water contains $2x10^8$ PFU of phages, and the total number of phages on earth is estimated to be $1x10^{30} – 1x10^{32}$. Phages are abundant in saltwater, freshwater, soil, plants and animals, and they even have been isolated from some vaccines and sera commercially available in the United States. Also, phages are commonly isolated from foods consumed by humans and other animals, they are normal commensals of the human body, and they have been commonly found in the human gastrointestinal tract, skin, urine, and mouth, where they are harbored in saliva and dental plaque. The abundance of phages in the environment – and the continuous exposure of humans to them – explains the extremely good tolerance of the human organism to phages. Indeed, during the approximately 80 years of therapeutic phage applications, phages have been administered to humans orally, in tablet or liquid formulations (10^5 - 10^{11} PFU/dose), rectally, locally (skin, eye, ear, nasal mucosa, etc.), in tampons, rinses and creams, as aerosols or intrapleural injections, and intravenously – and there have been no reports of serious complications associated with their use. Because of this apparent safety of phages, phage phi X174 has been used in the United States to monitor immune function in patients, including immunocompromised patients.

Phage preparations are currently being developed in the United States for use in animal husbandry and other agricultural settings, to control enteric pathogens and improve food safety. For example, phage cocktail PLSV-1 significantly reduces the incidence of *Salmonella* in broiler ceca, and phage LMP-102 significantly reduces levels of *L. monocytogenes* on various foods. In the human therapy area, the PhagoBioDerm preparation has been in trials in Georgia (one of the former Soviet Union republics) recently, showing reduction of MRSA in humans - a trial that exemplifies the potential value of phages to address untreatable or difficult-to-treat antibiotic resistant infections.

The mode of action of phages is due to specific and effective lysing of targeted bacteria; secondary mechanism of action may involve immune stimulation through the components of phage lysate. In this context, phages and phage-encoded enzymes are also utilized to kill preparations of bacteria for use as killed vaccines or to develop so called "bacterial ghost" vaccines. Immediate research is required to determine the value of presently used phage therapy in various pre-clinical and clinical settings (which, technologically, could be fairly rapidly adapted for clinical applications in the West). Also, phage-encoded lytic enzymes could be used as antibacterial agents (which may require a longer development period). Furthermore, phages and their encoded EPS-degrading enzymes may be active against bacterial biofilms and bacteria embedded in biofilms (e.g., *P. aeruginosa* and CF). Finally, lytic mechanisms of phages could be used to identify novel drug targets (which is the most long-term, but still very intriguing, approach).

The standard panel of controlled efficacy studies, pharmacokinetics, and compatibility with other interventions such as antibiotics, probiotics, etc. should be ascertained. The impact (short-term and long-term) on the normal microflora and interactions with bacterial biofilms remains unknown presently. The study of phage genomes and elucidating the role of various phage genes in the lytic cycle, and the study of the Phage-Bacteria interaction mechanisms and the pathways involved in the lytic cycle**,** may identify novel therapeutic targets or novel class of therapeutic agents.

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